pH Influences the Distribution of Microbial Rock-Weathering Phenotypes in Weathered Shale Environments

pH influences the distribution of microbial rock-weathering phenotypes in weathered shale environments

Samuels, T.[1*], Pybus, D.[2], Wilkinson, M.[3] & Cockell, C.S.[1]

1. UK Centre for Astrobiology, School of Physics and Astronomy, University of Edinburgh, UK

2. ICL Boulby, Boulby Mine, Cleveland, TS13 4UZ

3. Grant Institute, School of Geosciences, University of Edinburgh, UK

Correspondence:

t.samuels@ed.ac.uk

Toby Samuels,

1.54 Ashworth Laboratories,

Institute of Evolutionary Biology,

School of Biological Sciences

University of Edinburgh,

Charlotte Auerbach Road,

Edinburgh,

EH9 3FL

*Current address: Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, UK

ORCID ID: 0000-0001-9850-1230

ABSTRACT

Microbial rock weathering of shale forms an important part of global biogeochemical cycling and soil formation. Culture-independent analyses have revealed diverse microbial communities in weathered shale environments, yet few studies have attempted to discern the functional ecology of such communities in relatiFon to their rock weathering capabilities. In this study, phenotypic plate assays were used to determine the abundance of microbes with different rock weathering phenotypic traits in weathered shale environments. A physicochemical parameter (pH) is shown to influence the abundance of aerobic rock weathering microbes in weathered shale. Iron and manganese oxidizers were restricted to acidic environments, while siderophore producing and alkaline phosphatase producing microbes were largely confined to pH neutral environments. Furthermore, a clear separation in the spatial distribution of aerobic iron oxidizing and siderophore-producing microbes, as defined by a pH gradient across the sites sampled, was demonstrated. Phylogenetic analysis of isolates revealed that siderophore producing and alkaline phosphatase producing bacteria belonged to commonly identified rock weathering genera including *Arthrobacter*, *Pseudomonas* and *Streptomyces*. These results enhance our understanding how physicochemical parameters can define the composition and rock weathering potential of microbial communities.

KEY WORDS

Functional ecology, iron oxidizers, siderophore producers, microbial rock weathering, pH

INTRODUCTION

Shale rock comprises 25 % of the Earth's exposed continental landmass (Amiotte-Suchet et al. 2003) and forms two thirds of all sedimentary rock deposits (Ilgen et al. 2017). The weathering of shale in the natural environment therefore makes a significant contribution to global

biogeochemical cycles (e.g. C, Fe and S) and soil formation (Uroz et al. 2009; Brantley et al. 2012). The role of microbes in the weathering of shale has been extensively studied in natural environments (Petsch et al. 2001; Petsch et al. 2005; Joeckel et al. 2005; Cockell et al. 2011; Yesavage et al. 2012; Zhu and Reinfelder, 2012; Li et al. 2014; Włodarczyk et al. 2018). However, most of these studies have used culture-independent phylogenetic analysis and/or biogeochemical data to infer the impact of microbial activity on rock weathering processes, rather than directly quantify the rock weathering potential, or the phenotypic traits, of microbes present in those environments.

The phenotypic capabilities of rock weathering microbes have been of significant interest to those studying shale weathering within industrial contexts. For example, harnessing microbial activity within shale has been proposed to enhance metal recovery from low-grade ores (Anjum et al. 2012; Kutschke et al. 2015). Furthermore, microbial rock weathering activity has been indicated to increase environmental pollution from mining wastes (Wengel et al. 2006; Kalinowski et al. 2006) and to contribute to rock expansion in shale bedrock, causing significant damage to infrastructure (Anderson, 2008; Hoover and Lehmann, 2009). Such application-focused studies often quantify the biologically enhanced rate of elemental leaching from rock as a measure of the weathering potential of individual microbial strains (Tasa et al. 1997; Anjum et al. 2010) or microbial communities (Lee et al. 2005; Matlakowska et al. 2012; Włodarczyk et al. 2015). These studies have revealed numerous microbial rock weathering mechanisms including the oxidation of iron (Tasa et al. 1997, Grobelski et al. 2007; Spoalore et al. 2011) secretion of siderophores (Kalinowski et al. 2006, Włodarczyk et al. 2015) and organic acid production (Anjum et al. 2010; Włodarczyk et al. 2016) that result in the enhanced dissolution and degradation of shale.

Functional ecology is the study of the functional roles species play within their native ecosystem (Prosser et al. 2007). Within the context of geomicrobiology, one approach to

explore the functional ecology of weathered rock environments is through the use of agar plate phenotypic assays, which allow microbial isolates with specific capabilities to be identified (Hirsch et al. 1995). Microbial colonies grown on a defined agar type can be recorded as positive or negative for a phenotype based upon a visual test result (Schwyn and Neilands, 1987; Pérez et al. 2007). Matlakowska and Sklowdowska (2009) used this approach to characterise eight bacterial strains isolated from black shale, determining their ability to degrade a variety of recalcitrant organic compounds (e.g. phenanthrene), produce degradative enzymes (e.g. dioxygenases), to uptake and degrade metalloporphyrins, and to produce siderophores. As black shale is rich in sedimentary organic matter (2-15 % weight composition), the ability of shale-inhabiting organisms to degrade organic matter contributes to their ability to weather shale. Matlakowska and Sklowdowska (2009) demonstrated that while some capabilities were shared by most of the isolates (e.g. haem degradation), only one isolate was identified that could produce dioxygenases and degrade phenanthrene. From a microbial ecology perspective, it is interesting to consider if the differences in rock weathering potential between isolates can be explained by the environmental conditions in which those isolates were obtained. The rock weathering effect of microbes is known to be constrained by the physicochemical parameters of their surrounding environment (Calvaruso et al. 2006; Calvaruso et al. 2010). For example, as ferric iron is significantly less soluble at pH values above 4, the production of siderophores in pH neutral environments to increase iron bioavailability is advantageous to capable organisms (Kalinowski et al. 2006). In contrast, the bioenergetic favourability of aerobic iron oxidation is known to increase below pH 4 (Hedrich et al. 2011). Therefore, the abundance of siderophore-producing and iron oxidizing microbes across rock weathering environments could be predicted to be negatively correlated, but there is a lack of empirical evidence from culture-based studies to support this hypothesis.

93 Chen et al. (2016) investigated the variability of weathering potential across 150 bacterial
94 isolates obtained from across a weathered rock gradient (less-altered siltstone, more-altered
95 siltstone and soil). To quantify rock weathering potential of isolates, the authors quantified the
96 enhancement of elemental leaching from unaltered siltstone, the secretion of siderophores and
97 the release of organic acids in liquid-rock culture. They identified numerous differences in
98 these capabilities between isolates obtained across the weathered rock gradient, with isolates
99 taken from soils having a greater ability to leach iron, silicon and potassium from the rock
100 compared to isolates from less-altered rock. Interestingly however, the extent of siderophore
101 production (a rock-weathering trait) did not significantly vary between isolates taken from
102 across the weathered rock gradient (Chen et al. 2016).

103 In this study, we attempt to determine if rock surface pH influences the distribution of microbes
104 with rock weathering phenotypic traits, which could constrain the potential for microbial
105 communities to contribute to weathering processes. We chose six phenotypic plate assays that
106 enrich for: 1-2) isolates that grow on nutrient agar (at both neutral and acidic pH), 3)
107 siderophore producers, 4) alkaline phosphatase producers, 5) iron oxidizers and 6) manganese
108 oxidizers. These assays have been used to enumerate the presence of these phenotypic trait
109 groups in samples taken from across eroding shale cliffs and other weathered shale
110 environments on the North Yorkshire coastline, UK.

111 Coastal erosion of the rocky cliffs on the North Yorkshire coastline is a pervasive hazard and
112 poses substantial risk to infrastructure (Guthrie and Lane, 2007; Rosser et al. 2013). Recent
113 studies have obtained accurate rates of erosion (Lim et al. 2005; Rosser et al. 2007) and
114 attempted to determine which geomorphological processes constrain those rates (Lim et al.
115 2010; Van Jones et al. 2015). An important finding of this work is that cliff erosion at studied
116 sites was best described by progressive rock mass deformation, with rock falls of smaller
117 magnitude proceeding larger magnitude events (Lim et al. 2010). The role of rock weathering

processes, including microbial activity, which could accelerate the rate of these low magnitude events was explored in our previous work (Cockell et al. 2011; Samuels et al. 2019). This study has continued to use the North Yorkshire coastline to investigate microbial shale weathering activity, based upon the significant environmental and economic consequences that weathering processes potentially impact.

METHODS

Field sites and sample collection

Rock and water samples were obtained across nine different sites on the North Yorkshire coastline (Figure 1) in August 2015. The following is a list of these sites with their numeric ID: 1) cliff surface at Hole Wyke, a bay near to Boulby Head, 2) cliff surface revealed by a rock fall at Hole Wyke, 3) walls of jet mines located near Tellgreen, 4) cliff surface at Keldhowe Steel, 5) ochreous water from a stream within Deepgrove quarry, 6) outcrop surface at Sandsend Ness quarry 7) scree slope of Gaytres quarry, 8) walls from one of the levels of Assholm cement stone mines and 9) cliff surface at Saltwick Bay. These sites include a variety of natural weathered cliff outcrops (sites 1-2, 4 and 9) and historic industrial sites including quarries (5-7) and mines (3 and 8). The position of these sites, including that of the whole sampling area on the UK coastline, can be seen in figure 1; representative images of some of these field sites can be seen in figure 2.

Samples obtained in this study came from strata within two geological stages within the Early Jurassic period (Hobbs et al. 2012). Samples taken at sites 1 and 2 were from pyritous shale in the Redcar Mudstone Formation (RMF), deposited during the Pleinsbachian stage (182.7-190.8 Ma). All other samples were taken from two members (Alum shales and Mulgrave shales) of the Whitbian Mudstone Formation (WMF), deposited during the Toarcian stage (174.1-182.7 Ma). Samples taken from sites 3-6 and 7-9 were taken from the Mulgrave shale and Alum shale

members respectively. The mineralogy of both the RMF and WMF are similar, being largely comprised of quartz (24-31 %), mica (34-42 %) and kaolinite (16-21 %), with minor amounts of pyrite (2-5 %). All shales contained sedimentary organic matter in the form of type II kerogen (1.5-5.5 %), and are enriched in a number of metallic elements including iron and manganese (Hobbs et al. 2012). Further information on the geology and geochemistry of the sampling sites can be found in Samuels (2018) and selected references within (in particular, see Hobbs et al. 2012).

Sites were chosen based upon evidence of rock weathering at that site. Weathered surfaces of shale rock in these environments were heavily coated in red and purple mineral deposits that in appearance could be putatively identified as ferromanganese deposits (Figure 2), which are heavily comprised of iron and manganese oxides and hydroxides (Carmichael et al. 2013).

Previous geochemical characterisation of weathered shale on the North Yorkshire coastline (Cockell et al. 2011) demonstrated that weathered rock surfaces were coated in authigenic iron oxyhydroxides and localised acicular, platy and aggregated gypsum. Geochemical analyses of rock on the North Yorkshire coastline established the high abundance of both iron (5.88-11.04 %) and manganese (160-550 ppm) within the shale strata (Gad et al. 1968). Phylogenetic analysis of the microbial community inhabiting the rock surface revealed a single iron oxidizing microbial species (*Acidiferrobacter thiooxydans*) within a low-diversity microbial community dominated by Proteobacteria (Cockell et al. 2011).

Within each sampling site, multiple locations (i.e. different spots on a cliff surface) were chosen for sampling. The number of locations sampled at each individual site varied (2-5), based upon the availability of surfaces to sample: site 1 (2 locations), site 2 (3), site 3 (4), site 4 (2), site 5 (2), site 6 (2), site 7 (2), site 8 (5) and site 9 (3). A flame sterilised rock hammer was used to either break away or dislodge pieces of bulk rock or saprolites (chips of slaked, weathered rock)

166 from rock surfaces into Whirlpak© bags. Water from a stream running through Deepgrove
167 quarry was collected in 50 mL Falcon tubes. A water sample was included in this study due to
168 the abundance of ochre material visually identifiable within the water (Figure 2F), that
169 appeared to be weathered mineral products derived from the surrounding rock. Samples were
170 kept at ambient temperature until return to the University of Edinburgh, whereupon they were
171 stored at 4 ºC until use. Due to the large number of samples to be processed, it took one month
172 to finishing processing and plating out all samples. Storing samples at 4 ºC for a prolonged
173 period potentially affected microbial viability within the samples. However, the storage
174 conditions were the same for all samples, meaning they should have effected equally.

175 ## pH measurements

176 Within one month of collection, ferromanganese crusts on shale samples were scraped from
177 rock surfaces using a scalpel. Care was taken to only scrape surface coatings until un-weathered
178 rock below was revealed, to obtain the most representative estimate of rock surface pH. Rock
179 chips from all sampling locations within a site were pooled, due to the low mass of scrapings
180 that could be obtained from samples taken at any one sampling location. For each pH
181 determination, 50 μg of scrapings were suspended in 2 mL of Milli-Q filtered water and
182 allowed to equilibrate for 30 minutes. Triplicate measurements were then taken for each
183 equilibrated sample. For water samples collected at Deepgrove quarry, the pH of the sample
184 was measured directly. All pH values were converted into hydrogen ion concentrations for use
185 in the statistical analyses (see results, figure 4).

186 ## Phenotypic trait agar plate assays

187 To determine the abundance of aerobic, culturable microbes with a specific phenotype
188 inhabiting weathered shale, rock sample suspensions were plated onto petri dish plates
189 comprised of one of six different agar types. These agar types enabled the positive

identification of colonies with the following phenotypic traits: 1) heterotrophic growth on pH neutral media, 2) heterotrophic growth on acidic media, 3) siderophore production, 4) alkaline phosphatase production, 5) iron oxidation and 6) manganese oxidation. Each agar type allowed positive identification of microbial isolates with a specific phenotype, either through a visually recognisable color change or through the presence alone of microbial colonies on that agar type.

We choose to specifically study aerobic rock weathering organisms based upon their relevance to the sites sampled (primarily rock-air interfaces). Phenotypic traits were chosen based upon either their known importance in microbial rock weathering of shale (Kalinowski et al. 2006; Spoalore et al. 2011; Włodarczyk et al. 2015) or of rock weathering more generally (Uroz et al. 2009; Gadd, 2010; Carmichael et al. 2013), and based upon the availability of agar plate phenotypic assay methodology.

The authors appreciate that the agar types used in this study enrich for microbial isolates within narrow, specific metabolic/phenotypic groupings, rather than being broad, all-encompassing assays. For example, the iron oxidizers enriched in this study are those isolates that can specifically grow on WAYE agar (below and Table 1), meaning they are at least acidotolerant, if not acidophilic. Furthermore, we cannot comment whether these organisms are autotrophic or heterotrophic iron oxidizers, as the medium contains nutrients that would facilitate growth and activity of both groups. Finally, as all incubations carried out in this study were under aerobic conditions, our culturing approach could not identify anaerobic or microaerophilic iron oxidizers.

With the exception of nutrient agar based media (both pH neutral and acidic media were used), all phenotypic groups were isolated within a single pH range. For example, the CAS agar plates (used to detect siderophore production) had a media pH of 6.8. This limitation could not be

overcome by preparing media at differing pH levels (e.g. pH neutral and acidic CAS agar), as the chemical reactions that provide a visual result in these media types are pH sensitive (e.g. acidified CAS agar will not be functional). Further methodological limitations apply to the media used to enrich for alkaline phosphatase producers, manganese oxidizers and iron oxidizers. Despite these issues, our approach enabled the isolation of geomicrobiologically-relevant microbial groups from our sample sites. Throughout this manuscript, the microbial groups isolated in this study are referred to by their general name for ease (e.g. aerobic, acidotolerant/acidophilic iron oxidizers are called "iron oxidizers").

No attempt to distinguish between bacteria, fungi or archaea was made, however only successfully amplified 16S rDNA sequences from bacterial isolates were submitted for sequencing (see below, Colony PCR and Sanger sequencing). All agar types contained an added source of organic carbon, in addition to organic material within the shale rock supplement, therefore both autotrophic and heterotrophic isolates could be cultivated on all media types and could not be distinguished from each other with the methods used. Brief details of each agar type are included in Table 1, with media recipes detailed in the "Agar media" section below. Further details on each agar type can be found in the provided selected references.

One gram aliquots of samples from each location (25 locations across 9 field sites) were suspended in 5 mL of 1x PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4, 1 L water - adjusted to pH 7 with HCl) in 50 mL Falcon tubes and hand inverted for 5 minutes to ensure thorough mixing. Suspensions were serially diluted in phosphate buffered saline (PBS) and plated out onto each of six agar types (see media below) in triplicate. This created a total of 75 plates (triplicate plates for 25 samples) for each agar type. Plates were wrapped in parafilm and incubated aerobically at room temperature (~23 ^{o}C) for one month, and the number of colonies positive for that phenotype recorded. These colony counts were then

adjusted to create CFU g^{-1} values for each phenotype within a sample. Un-inoculated plates and plates inoculated with just PBS were incubated alongside sample plates as sterile controls.

Agar media

All agar media recipes are for 1 L preparations made with Milli-Q UV- purified and filtered (0.22 µm) water, and autoclaved at 121 ºC for 20 minutes unless otherwise specified. All agar media was supplemented with 2 g L^{-1} of black shale rock powder obtained by collection and subsequent crushing of (<0.5 mm size fractionated) slabs of black shale from the Mulgrave shale member at Saltwick Bay.

Nutrient agar: 28 g nutrient agar powder, 2 g rock powder.

Acidic nutrient agar: Solution A contains 28 g nutrient broth powder and 2 g rock powder mixed in 750 mL of water, which is then adjusted to pH 3 with H_2SO_4. Solution B is prepared by mixing 7 g agarose in 1 L of water and stirred for 30 minutes to allow agarose hydration. The agarose was then allowed to settle, and 750 mL of the water removed, leaving a remaining 250 mL agarose suspension. Both solutions A and B are autoclaved and then mixed while still molten.

Chrome Azurol S (CAS) blue agar: Solutions A, B and C were prepared as follows, A) 0.324 g $FeCl_3 \cdot 6H_2O$ was mixed in a 100 mL aqueous solution of 0.01 M HCl, B) 60.5 mg of CAS powder in 50 mL of water and C) 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) in 40 mL of water. Ten millilitres of solution A was mixed with all of solution B, which was then mixed with solution C, creating solution D. Solution E is prepared from 200 mL of 5x M9 salts (34 g Na_2HPO_4, 15 g KH_2PO_4, 2.5 g NaCl and 5.0 g NH_4CL in 1 L water - autoclaved), 15 g agar, 30.25 g piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES) and 2 g of rock powder, mixed in 650 mL of water and adjusted to pH 6.8 with NaOH. Both solutions D

and E were autoclaved, and then allowed to cool to 50 ^{o}C in a water bath before being mixed to create the final media.

Phenolphthalein phosphate agar: 15 g of Phenolphthalein phosphate agar (PPA, purchased from Sigma-Aldrich) and 2 g of rock powder.

Manganese oxidizer agar: 2 g Peptone, 0.5 g yeast extract, 1 mg $FeSO_4 \cdot 7H_2O$, 150 mg $MnSO_4 \cdot H_2O$, 2.38 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 8 mg LBB, 2 g rock powder and 15 g agar were added to 1 L water and adjusted to pH 7.4 using NaOH/HCl.

Washed Agarose - Yeast Extract (WAYE) agar: A basal salts solution (1 L - autoclaved) was prepared by the addition of 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g $(NH4)_2SO_4$, 1 g KCl and 0.1 g of $Ca(NO_3)_2 \cdot 4H_2O$. Solution A was prepared by mixing 100 mL of basal solution to 650 mL of water, with 0.2 g of yeast extract powder and 2 g of rock powder added. This solution is then adjusted to pH 3 with sulfuric acid before autoclaving. Solution B is prepared by mixing 7 g agarose in 1 L of water and stirred for 30 minutes to allow agarose hydration. The agarose was then allowed to settle, and 750 mL of the water removed. The remaining 250 mL containing the hydrated agarose is then autoclaved. Solution C) 800 mM $FeSO_4 \cdot 7H_2O$, adjusted to pH 3 with sulfuric acid before filter sterilization (0.22 µm filter) was prepared. When hot, solutions A and B are mixed (ensuring agar is fully molten) along with 5 mL of solution C under sterile conditions.

Colony PCR and Sanger sequencing

Forty six colonies were picked from phenotypic assay plates, based upon visual assessment of unique colony morphology, for phylogenetic identification of the microbial isolates. Isolates that were obviously fungal in morphology were not selected, as only 16S rDNA sequencing to identify bacterial species was carried out. Selected individual colonies were picked and

suspended in 50 µL of molecular grade water. One microlitre of this solution was used as the DNA template for the PCR reaction. Each 25 µL reaction included a 12.5 µL PCR Mastermix, 1 µL of both the forward and reverse primer solutions and 10.5 µL of molecular grade water. PCR Mastermix (New England Biolabs) contained Taq DNA Polymerase, deoxynucleoside triphosphates (200 µM) and buffer ($MgCl_2$ 1.5 mM). Primer concentration was 0.4 µM in the final PCR reaction volume.

The forward primer used was 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer used was 1389R (5'-ACGGGCGGTGTGTACAAG-3'). The PCR procedure started with a 10 minute denaturation step at 96° C for 10 minutes, followed by 30 cycles of denaturation at 95° C for 1 minute, annealing at 60° C for 1 minute and extension at 72° C for 1 minute, with a final step of extension at 72° C for 10 minutes. A negative control lacking added DNA template was also performed to rule out false positive results from reagent contamination. PCR products were analysed using gel electrophoresis.

Successfully amplified products were processed using a DNA Purification Kit (Qiagen) according to the manufacturer's instructions, and analysed by Sanger sequencing at Edinburgh Genomics with the same primer set used for PCR reactions. Processed reads (quality control checked and trimmed) were provided by Edinburgh Genomics, which were then aligned and forward and reverse sequences stitched to produce contigs using the software DNA Sequence Aligner (v4) from DNA Baser. Contigs were then aligned in the alignment, classification and tree (ACT) service of ARB-Silva (Pruesse et al. 2012), allowing neighbour sequences with >97% similarity to be obtained and isolate identity to be determined (Quast et al. 2013; Yilmaz et al., 2014; Glöckner et al. 2017). Isolate identifications were taken from the European Molecular Biology Laboratory (EMBL) database within ACT. ACT was also used to produce a .tree file (Beccati et al. 2017) which was visualized in iTOL (Letunic and Bork, 2016).

Cultured isolates are labelled on the phylogenetic tree with the following label structure: phenotype (SP/APP)_ site number (e.g. 1)_ unique identifier.

Data and statistical analysis

Basic data manipulation and statistical analyses were carried out in R (version 3.5.3) (R Core Team, 2019). A Spearman's Rank Correlation analysis was performed on CFU g^{-1} data and pH values to determine if significant positive and/or negative correlations occurred between phenotypic trait groups and with pH. Figures were produced using the R packages ggplot2 (Wickham, 2016) and corrplot (Wei and Simko, 2017).

RESULTS

Isolates cultured on nutrient agar were identified in samples collected from all nine sampling sites, ranging in abundance between 10^3 and 10^7 CFU g^{-1} (Figure 3). This was the only phenotype tested for that was identified in samples from all sampling sites. In contrast, isolates cultured on acidic nutrient agar were only identified in samples taken from four sites, three of which were identified as highly acidic (< pH 3) from pH measurements taken of rock surface scrapings (Figure 3).

All phenotypes screened for were identified in samples from at least one site (Figure 3). Alkaline phosphatase producers were widely distributed among the samples collected, with abundance ranging across two orders of magnitude ($5 \times 10^3 - 3 \times 10^5$ CFU g^{-1}) from eight of nine sites sampled (Figure 3). Manganese oxidizing isolates (identified at sites 6, 7 and 9 only) were the least distributed among the sites sampled (Figure 3).

Interestingly, manganese oxidizers were isolated from sites (6, 7 and 9) below pH 3 (Figure 3), despite the fact the media used to culture these isolates was circumneutral (Table 1). This indicates that the manganese oxidizers isolated in this study could tolerate a wide pH range,

and that media pH did not constrain the enrichment of manganese oxidizers from sample sites with a differing pH. It should be noted that "tolerate" in this context refers to the viability of the microbes within the sample, and does not indicate the extent of their activity (active vs dormant) within the environment.

Samples taken from highly acidic (< pH 3) sites yielded positive isolates for the most number of phenotypes tested for, with phenotypically positive isolates cultured from samples 6, 7 and 9 being found on five out of six of the agar types tested (all except siderophore producers). In comparison, samples collected from sites 1-4, which had moderately alkaline (~ pH 8) to moderately acidic (pH 5.5-7) rock surfaces yielded phenotypically positive isolates for only 2-3 of the phenotypes tested for (nutrient agar isolates and alkaline phosphatase producers in all cases) (Figure 3).

Histograms of CFU g^{-1} abundance data for each tested phenotype demonstrated that the data collected was not normally distributed, meaning that a non-parametric (Spearman's rank-order correlation) analysis was required to test for associations between each of the six groups and with the environmental parameter pH (Figure 4). Eight associations were identified as significant at the 0.99 confidence level, with a further 3 significant associations identified at both confidence levels of 0.95 and 0.9 respectively (total associations=14) (Figure 4).

pH significantly correlated with all six phenotypic trait groups, with positive associations identified with iron oxidizers (r_s=0.45, $p<0.01$), acidic nutrient agar isolates (r_s=0.55, $p<0.01$) and manganese oxidizers (r_s=0.46, $p<0.01$), and negative associations identified with nutrient agar isolates (r_s=-0.3, $p=0.01$), siderophore producers (r_s=-0.46, $p<0.01$) and alkaline phosphatase producers (r_s=-0.22, $p=0.06$). These two sets of associations with pH are internally supported by further positive correlations between the abundances of the phenotypes within each set (Figure 4). For example, the abundance of iron oxidizers positively correlated with the

abundance of both manganese oxidizers (r_s=0.79, p<0.01) and acidic nutrient agar isolates (r_s =0.57, p<0.01). Positive correlations between those phenotypes that negatively correlated with pH were weaker but still significant, such as the correlation between siderophore producers with both alkaline phosphatase producers (r_s=0.27, p<0.05) and nutrient agar isolates (r_s=0.21, p=0.07). Furthermore, two negative correlations in the abundance of phenotypes between the pH association sets was identified, with siderophore producers negatively correlating with iron oxidizers (r_s=-0.26, p<0.05) and manganese oxidizers (r_s=-0.19, p<0.1).

Twenty two isolates were successfully sequenced and the genus of the organism identified (Figure 5, Table 2). The result of this analysis was the identification of eight different genera across two phenotypic trait groups. These eight genera were from a diverse range of phyla, including Actinobacteria, Firmicutes, α-Proteobacteria and γ-Proteobacteria (Figure 5, Table 2).

Roughly equal numbers of isolates from each of the weathering phenotypes processed for sequencing were identified: 10 siderophore producers and 12 alkaline phosphatase producers. Strains of *Aeromonas, Rhizobium* and *Psueudomonas* were identified within both phenotypic groups. Of the 10 siderophore producing isolates successfully sequenced, eight belonged to the genus *Pseudomonas* (Figure 5, Table 2).

No phylogenetic data for the iron and manganese oxidizing colonies could be obtained in this study. Initial difficulty was encountered in performing colony PCR with iron and manganese oxidizing colonies, however in some cases this was overcome by culturing those isolates on acidic nutrient agar. However, those successfully amplified 16S rDNA amplicons failed in subsequent sequencing reactions. The reason for these technical difficulties is unknown, but metal content bound to the biomass/nucleic acids could have affected reaction success.

DISCUSSION

381 The abundance of microbes within six phenotypic trait groups from samples of weathered shale
382 was determined. As microbial isolates were likely cultured on more than one of the six agar
383 types (e.g. siderophore producing microbial species were also likely to grow on nutrient agar),
384 combined with the fact that most microbial species in environmental samples are known to be
385 non-culturable (Kirk et al. 2004), an accurate assessment of the total biomass within the
386 samples collected was not made. However, the abundance of nutrient agar and acidic nutrient
387 agar isolates have been used in this study to provide a baseline for total biomass within the
388 samples of weathered shale (Puente et al. 2009). Nutrient agar isolates were cultured from all
389 nine sites sampled, ranging in abundance between 3 x 10^3 and 1.5 x 10^7 $CFUg^{-1}$, while acidic
390 nutrient agar isolates were identified at four sites and ranged in abundance from 1.3 x 10^3 and
391 4.4 x 10^5 CFU g^{-1} (Figure 3). This range of microbial abundance in samples of weathered shale
392 is consistent with a previous assessment made by Cockell et al. (2011), who used extractable
393 DNA content to estimate a total microorganism abundance of 4.2 x 10^4 cells cm^{-2}.

394 The occurrence of the remaining phenotypic trait groups (siderophore producers, alkaline
395 phosphatase producers, iron oxidizers and manganese oxidizers) varied substantially between
396 field sites (Figure 3). For example, at sites 1, 2 and 4 isolates were cultured from only two
397 phenotypic trait groups, nutrient agar isolates and alkaline phosphatase producers. In
398 comparison, sites 6, 7 and 9 yielded isolates from five of the six phenotypic trait groups (Figure
399 3). These differences can be qualitatively explained by differences in rock surface pH values,
400 with sites 1, 2 and 4 being pH neutral to moderately acidic (pH 5.8-7.7) while sites 6, 7 and 9
401 were strongly acidic (pH 2.6-2.8) (Figure 3). The low pH rock surface values obtained in this
402 study are consistent with the analysis of weathered shale crusts previously studied (pH 3.5,
403 Cockell et al. 2011).

404 To quantitatively determine if the abundance of isolates within phenotypic trait groups
405 correlated with rock surface pH, Spearman's rank correlation coefficient analysis was used

406 (Figure 4). Correlation analyses were also run for each pair combination of phenotypic trait
407 groups (e.g. iron oxidizers with siderophore producers) to determine abundance co-associations
408 between groups (Figure 4). pH was found to significantly influence the abundance of all six
409 phenotypic trait groups enumerated in this study, with the set of acidic nutrient agar isolates,
410 iron oxidizers and manganese oxidizers correlating with acidic pH values (r_s value range 0.45
411 to 0.55), and the set of nutrient agar isolates, siderophore producers and alkaline phosphatase
412 producers correlating with neutral to mildly acidic pH values (r_s value range -0.22 to -0.46)
413 (Figure 4). This control of pH over phenotypic trait abundance was supported by further
414 positive correlations between phenotypic trait groups within sets (e.g. iron oxidizers with acidic
415 nutrient agar isolates, r_s=0.57, $p<0.01$) and negative correlations between phenotypic trait
416 groups between sets (e.g. iron oxidizers with siderophore producers, r_s=-0.26, $p<0.05$) (Figure
417 4).

418 The association of aerobic, acidotolerant/acidophilic iron oxidizers with acidic environments
419 in this study (Figure 4) is unsurprising, as the bioenergetic favourability of aerobic microbial
420 iron oxidation is known to be restricted below pH 4 (Hedrich et al. 2011). Previous studies
421 have shown that microbial iron oxidation in shale is predominated by the oxidation of pyrite
422 (FeS_2), in which both the iron and sulfur become oxidized (Tasa et al. 1997; Joeckel et al. 2005;
423 Li et al. 2014). The oxidation of pyritic sulfur to sulphuric acid lowers environmental pH,
424 which in turn facilitates the growth of iron oxidizing microbes and the continued dissolution
425 of pyrite (Vera et al. 2013, but see also Samuels et al. 2019). As such, the interaction of iron
426 oxidizing and sulfur oxidizing microbial activity generates a positive feedback loop that
427 maintains a suitable habitat (< pH 4) for iron oxidizing microbes.

428 Siderophore production is known to of greater adaptive significance in pH neutral
429 environments where ferric iron bioavailability is low (Kalinowski et al. 2006), which explains
430 why siderophore producers were largely restricted to pH neutral environments in weathered

shale samples (Figure 3). These opposing relationships of iron oxidizers and siderophore producers with pH are further supported by the negative correlation between these phenotypic trait groups (r_s=-0.26, $p<0.05$), demonstrating that the approach we have used can effectively distinguish between rock weathering niches along a physicochemical gradient (Figures 3 and 4). As far as the authors are aware, this is the first empirical evidence of the spatial separation of aerobic iron oxidizers and siderophore producers across a pH gradient in rock weathering environments.

Unlike microbial iron oxidation, microbial manganese oxidation is not known to be limited to acidic environments, with the recommended pH value for the cultivation of manganese oxidizers ranging between pH 7-8 (Nealson, 2006). Despite this, the abundance of manganese oxidizers correlated positively with acidic environments (r_s=0.46, $p<0.01$), and with both iron oxidizers (r_s=0.79, $p<0.01$) and acidic nutrient agar isolates (r_s=0.57, $p<0.01$). A potential explanation for our findings is that the manganese oxidizing organisms isolated in this study have the ability to oxidize multiple metallic elements (e.g. iron and manganese) (Corstjens et al. 1992), and therefore are not actively oxidizing manganese in the acidic environments they were isolated from. However, as the rate of abiotic manganese oxidation of manganese is relatively slow compared to microbially-facilitated oxidation, and that the formation of manganese oxide deposits is believed to mainly biologically mediated (Tebo et al. 2004), it seems likely that the manganese oxide deposits identified at our field sites (Figure 2) are at least the partial result of microbial activity. An alternative hypothesis for our results is that the organisms isolated here may not be active oxidizers, i.e., they do not use enzymes such as multicopper oxidases to facilitate manganese oxidation (Geszvain et al. 2012), but that biomass of these organisms passively acts as a nucleation site for manganese oxidation (Tebo et al. 2005; Gadd, 2010). Further work, including phylogenetic identification and characterisation of

manganese oxidizing activity, would be required to resolve the exact role of the manganese oxidizing microbes isolated in this study in their natural environment.

The pH of the individual media types used in this study potentially contributed to the constraints on the distribution of the phenotypic trait groups enriched on those media types. For example, aerobic acidotolerant iron oxidizers were isolated from three sites that ranged in average surface pH from 2.6 to 5.2 (Figure 3). As the media used to enrich for this group (WAYE agar) was at pH 3, aerobic iron oxidizers from pH neutral sites that could not tolerate this pH were unlikely to be enriched. This is an inherent limitation of a culture-based approach to investigate the distribution of microbial groups, however we believe that in the case of our results this limitation likely had a minimal impact. The manganese oxidizers isolated in this study were cultivated on a pH neutral agar, but were entirely isolated from acidic sites, while siderophore producers cultivated on pH neutral agar were isolated from sites ranging in pH from 5.20 to 8.07. Finally, alkaline phosphatase producers were isolated from eight of the nine sites sampled, including from the most acidic and alkaline sites (Figure 3). Therefore, although media pH may have partially constrained our results, it does not explain the pattern and general trends we have observed.

The phenotypic trait agar plate assay used in this study to enumerate phosphate solubilizers tested for the production of alkaline phosphatases, which have been previously indicated in inorganic phosphate solubilisation in neutral and alkaline environments (Hughes and Lawley, 2003; Sharma et al. 2013). The correlation between the abundance of alkaline phosphatase producing microbes and neutral pH environments in this study can therefore be expected (Figure 4). In future work it would be interesting to compare the abundance of microbial isolates that produce acid and alkaline phosphatases, and determine if pH influences their distribution in rock weathering environments. In addition, determining the distribution of

anoxic and anaerobic microbial groups that contribute to microbial rock weathering in shale, such as neutrophilic iron oxidizers, would be a logical progression of this work.

Phylogenetic identification of the isolates cultured in this study was attempted for four phenotypic trait groups (not including nutrient agar and acidic nutrient agar isolates), but was only successful for siderophore producers and alkaline phosphatase producers. In total 22 isolates were successfully identified, revealing eight genera across four different phyla (Table 2, Figure 5). A single alkaline phosphatase producing isolate was identified as *Moraxella* (γ-Proteobacteria), which is the first time this genus has been associated with weathered shale environments. The genera of the remaining isolates have been previously identified in weathered shale, either through isolation from culture-based studies (e.g. Jiang et al. 2015) or in culture-independent analyses (e.g. Włodarczyk et al. 2018). Furthermore, most of these genera are well-known rock weathering organisms such as *Arthrobacter* (Frey et al. 2010), *Bacillus* (Puente et al. 2004), *Pseudomonas* (Matlakowska and Sklodowska, 2009), *Rhizobium* (Zhao et al. 2013) and *Streptomyces* (Cockell et al. 2013). In most cases, these organisms have been previously recognised to have the rock weathering phenotypic traits that they are associated with in this study, such as the siderophore production of *Pseudomonas* (Kalinowski et al. 2006) and *Rhizobium* (Zhao et al. 2013), or the phosphate solubilisation of *Bacillus* (Puente et al. 2004) and *Streptomyces* (Hamdali et al. 2008). It is therefore highly likely that the organisms identified in this study are active rock weathering organisms within weathered shale environments.

CONCLUSIONS

In this study, the abundances of microbes with differing phenotypic traits were found to be influenced by a physicochemical parameter (pH). Iron and manganese oxidizing microbes were restricted to acidic environments, while siderophore producing and alkaline phosphatase

503 producing microbes were largely constrained to pH neutral environments. Furthermore, the
504 first empirical evidence for a clear separation of the distribution of aerobic iron oxidizers and
505 siderophore producers in rock weathering environments was shown, which can be readily
506 explained by changes in iron geochemistry across pH gradients. Phylogenetic analysis of
507 siderophore producing and alkaline phosphatase producing bacterial isolates revealed several
508 commonly identified rock weathering genera, indicating that these organisms are likely active
509 rock-weathering organisms in their native environments.

510 Our results show that physicochemical parameters can be used to predict the distribution of
511 functional groups. Future research should attempt to discern the effect physicochemical
512 parameters have on the expression and efficacy of rock weathering phenotypic traits within
513 these groups. Combined, this knowledge could be used to better estimate the contribution of
514 microbial communities to rock weathering rates in the field (Yesavage et al. 2012; Ilgen et al.
515 2017). Finally, our study demonstrates the importance of culture-based approaches to
516 investigate the functional ecology of microbial communities.

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